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4. PERFORMING ORGANIZATION REPORT NUMBER(S)

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
U.S. Army Medical Research Institute of Infectious Diseases	SGRD-UIS-C	U.S. Army Medical Research and Development Command

6c. ADDRESS (City, State, and ZIP Code)	7b. ADDRESS (City, State, and ZIP Code)
Fort Detrick, Frederick, MD 21701-5011	Fort Detrick, Frederick, MD 21701-5012

8a. NAME OF FUNDING/SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.

DTIC ELECTED 23 FEB 1989 S E

11. TITLE (Include Security Classification)

Hepatic Subcellular Distribution of [^{3}H]T-2 Toxin

12. PERSONAL AUTHOR(S)

Judith G. Pace and Michael R. Watts

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Interim	FROM _____ TO _____	10 February 1989	18

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

hepatic, subcellular, T-2, distribution

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

J. G. PACE and M. R. WATTS. Hepatic subcellular distribution of [^{3}H]T-2 toxin. *Toxicon* , - 19 . The subcellular distribution of T-2 mycotoxin and its metabolites was studied in isolated rat livers perfused with [^{3}H]T-2 toxin. After a 120-min perfusion, the distribution of radiolabel was to bile (53%), perfusate (38%), and liver (7%). Livers were fractionated into mitochondria, endoplasmic reticulum (smooth and rough), plasma membrane, and nuclei. Plasma membrane fractions contained 38% of the radiolabel within 5 min, decreasing to < 1% at the end of the 120-min perfusion. Smooth endoplasmic reticulum contained 27% of the radiolabel by 5 min and increased to 43% over the 120-min perfusion. The mitochondrial fraction contained 3% of the radiolabel by 30 min and increased to 10% after 120-min perfusion. Label in the nuclear fraction remained constant at 7%.

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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
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21. ABSTRACT SECURITY CLASSIFICATION

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

from 30 to 120 min. By 15 min, only the parent toxin was detected in the mitochondrial fraction. In the other fractions, radiolabel was associated with HT-2, 4-deacetylneosolaniol, T-2 tetraol, and glucuronide conjugates. Glucuronide conjugates accounted for radiolabel eliminated via the bile. The time course for distribution of radiolabel in liver suggested an immediate association of [^{3}H]T-2 with plasma membranes and a subsequent association of toxin and metabolites with endoplasmic reticulum, mitochondria, and nuclei, the known sites of action of this toxin.

HEPATIC SUBCELLULAR DISTRIBUTION OF [^{3}H]T-2 TOXIN

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Running Title: Subcellular Distribution of T-2

*Portions of the work have been presented in abstract form

(Toxicon, 5, 675, 1983)

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences-National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the author(s) do not purport to reflect the positions of the Department of the Army or the Department of Defense.

J. G. PACE and M. R. WATTS. Hepatic subcellular distribution of [^{3}H]T-2 toxin. *Toxicon* , - 19 . The subcellular distribution of T-2 mycotoxin and its metabolites was studied in isolated rat livers perfused with [^{3}H]T-2 toxin. After a 120-min perfusion, the distribution of radiolabel was to bile (53%), perfusate (38%), and liver (7%). Livers were fractionated into mitochondria, endoplasmic reticulum (smooth and rough), plasma membrane, and nuclei. Plasma membrane fractions contained 38% of the radiolabel within 5 min, decreasing to < 1% at the end of the 120-min perfusion. Smooth endoplasmic reticulum contained 27% of the radiolabel by 5 min and increased to 43% over the 120-min perfusion. The mitochondrial fraction contained 3% of the radiolabel by 30 min and increased to 10% after 120-min perfusion. Label in the nuclear fraction remained constant at 7% from 30 to 120 min. By 15 min, only the parent toxin was detected in the mitochondrial fraction. In the other fractions, radiolabel was associated with HT-2, 4-deacetylneosolaniol, T-2 tetraol, and glucuronide conjugates. Glucuronide conjugates accounted for radiolabel eliminated via the bile. The time course for distribution of radiolabel in liver suggested an immediate association of [^{3}H]T-2 with plasma membranes and a subsequent association of toxin and metabolites with endoplasmic reticulum, mitochondria, and nuclei, the known sites of action of this toxin.

INTRODUCTION

T-2 mycotoxin (4β,15-diacetoxy 3α-hydroxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene) is a potent inhibitor of protein synthesis in eukaryotic cells (UENO *et al.*, 1968) and isolated mitochondria (PACE *et al.*, 1988) and inhibits mitochondrial respiration (PACE, 1983; SCHILLER and YAGEN, 1981). TRUSAL and O'BRIEN (1986) found that the morphology of rough endoplasmic reticulum and mitochondria was altered by exposure to the toxin. MIDDLEBROOK and LEATHERMAN (1989) have studied the transport mechanism of T-2 toxin in cell systems and others have investigated the toxin binding to isolated ribosomes (BARBACID and VAZQUEZ, 1974; CARTER and CANNON, 1978; McLAUGHLIN *et al.*, 1977; MIDDLEBROOK and LEATHERMAN, 1989). Most data support a permeable membrane model for T-2-cell association rather than a receptor/transport system (MIDDLEBROOK and LEATHERMAN, 1989).

While the *in vitro* metabolic pathway of T-2 toxin and the subcellular localization of the participating enzymes have been reported by YOSHIZAWA *et al.* (1982, 1984), little information is available on *in vivo* toxin-subcellular interactions such as binding to other organelles and metabolism. The isolated perfused liver is an ideal model for studying toxin-cell interactions, detoxication and elimination of T-2 toxin (PACE, 1986). In this study we define the time-dependent association of T-2 toxin and its metabolites with hepatic-subcellular organelles isolated from rat livers perfused with an LD_{50} of [^{3}H]T-2.

MATERIALS AND METHODS

Livers from fasted-male, Fischer rats (Charles River Laboratories, Wilmington, MA), weighing 250-300 g, were perfused as described by PACE (1986), except that the perfusate was recirculated. After 30 min of equilibration, 50 µl of a 10 mg/ml solution of [^{3}H]T-2 (New England Nuclear, Boston, MA; sp act 8.9 Ci/mmol) was added to 125 ml of perfusion medium. The rate of toxin delivery to liver was 40 µg/min. The bile and effluent perfusate were collected at 5-min intervals. After 5, 15, 30, 60, or 120 min the livers were rapidly excised and homogenized in a chilled glass/Teflon homogenizer containing 10 ml of ice-cold homogenization medium (210 mM mannitol, 60 mM sucrose, 10 mM KCl, 10 mM sodium succinate, 1 mM ADP, 0.25 mM dithiothreitol and 0.1 mM EGTA in 10 mM Hepes/KOH buffer, pH 7.4). Liver homogenates were fractionated on Percoll step-gradients as described by REINHART *et al.* (1982). The total preparation time was 5-6 min. All operations were performed at 0-4°C.

Buffers and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Protein was measured by the Lowry method (LOWRY *et al.*, 1951). The distribution of organelles was determined by marker enzymes as described by REINHART *et al.* (1982). Bile (5 µl), perfusate (100 µl), and fractions from the Percoll gradient (100 µl) were analyzed for total radioactivity in a scintillation counter (Beckman Instruments Inc., Columbia, MD). Radioactivity per fraction was adjusted for a 0-time T-2-distribution control and normalized to 100% specifically bound toxin. Recovery of radiolabel was 98±2%. Results are expressed

as mean ± SEM for data obtained from three separate perfusions per time point (n=3). Aliquots of pooled-Percoll gradient fractions (20 µl), perfusate (50 µl) or bile (5 µl) were chromatographed on silica gel thin-layer chromatography plates and analyzed for T-2 and metabolites as described by PACE *et al.* (1985).

RESULTS

After 120 min, total delivered radiolabel was distributed to bile (53%), perfusate (38%), and liver (7%). Table 1 shows T-2 in the perfusate was completely converted to more polar metabolites by 60 min. Glucuronide conjugates of HT-2 and metabolites of T-2 were detected in bile at all time points (data not shown).

The distribution of protein and marker enzymes in fractionated liver homogenates was similar to that reported by REINHART *et al.* (1982) (Table 2). The fractions were identified by marker enzymes as: fraction 1, soluble enzymes; fraction 2, smooth endoplasmic reticulum (SER); fractions 3 and 4, plasma membranes; fractions 5 and 6, rough endoplasmic reticulum (RER); fraction 7, peroxisomes, lysosomes, and mitochondria; fraction 8, mitochondria; and fraction 9, nuclei. Table 2 shows the distribution of total radioactivity in each of the nine fractions over time. At 5 min, most of the radiolabel was associated with soluble proteins, SER and plasma membranes. Over 120 min of perfusion, there was a gradual increase in radiolabel associated with the SER, RER, and mitochondria-rich (fraction 8) fractions.

Figure 1 shows the percent distribution of T-2 and metabolites in fractions of liver homogenates at 5 and 120 min. At 5 min, membrane-bound radiolabel (fraction 4) was evenly distributed to T-2 and metabolites (HT-2, 4-deacetylneosolaniol (4DN), T-2 tetraol and glucuronide conjugates). Fractions 1 and 3 contained mainly 4DN, T-2 tetraol and conjugates, while fraction 2 (SER) contained mainly T-2 tetraol. Equal amounts of T-2, HT-2, and

4DN were associated with the nuclear fraction (fraction 9). By 120 min, all radiolabel found in the mitochondria-rich fraction (fraction 8) was in the form of T-2 toxin. Less than 1% of the radioactivity was found in the membrane fractions (fractions 3 and 4) and that associated with smooth (fraction 2) or rough (fractions 5 and 6) ER was in the form of polar metabolites and conjugates.

DISCUSSION

T-2 toxin-subcellular interactions *in vivo* are potentially significant to the understanding of the mechanism of action of this toxin. Development of a method to fractionate liver homogenates quickly into definable subfractions (REINHART *et al.*, 1982) and the availability of radiolabeled toxin have enabled us to study the hepatic distribution time course of T-2 and its metabolites.

The data presented in Table 2 and Fig. 1 indicate that at 5 min, 90% of the total cell-associated radioactivity appeared in the soluble, SER and plasma membrane fractions. While there was an early association of the parent toxin with the plasma membrane fractions (fraction 3 and 4), the majority of the counts were in the form of T-2 tetraol and glucuronide conjugates of T-2 metabolites. Thus, it is apparent that T-2 was rapidly metabolized by microsomal enzymes and that the portion of radiolabel that remained in the liver was in a more polar form.

Although ribosomal binding and subsequent protein synthesis inhibition have been studied in detail as a site and mechanism of action of T-2 toxin (UENO *et al.*, 1975; McLAUGHLIN *et al.*, 1977; CARTER and CANNON, 1978; THOMPSON and WANNEMACHER, 1984), little parent toxin was found in these fractions. The more polar metabolites (other than HT-2) that were distributed to ribosomes and RER are 10-200-fold less toxic to isolated hepatocytes (THOMPSON and WANNEMACHER, 1986). *In vivo* toxicity, however, appears to correlate more with the rate of uptake of T-2 and its metabolites (MIDDLEBROOK and LEATHERMAN, 1989) since, once bound

to ribosomes, all T-2 metabolites compete equally for binding of parent toxin and similarly inhibit protein synthesis. Therefore, it would appear that the total radiolabel (T-2 plus metabolites) associated with ribosomal fractions is more significant than the parent T-2-association.

Only 2-10% of the radioactivity was distributed to the mitochondria-rich fraction (fraction 8). However, at every time point, this radiolabel was all in the form of T-2 toxin. The accumulation of parent toxin in the mitochondria fraction could result from the inability of that organelle to metabolize the toxin or from a selectivity in its toxin/metabolite uptake mechanism. These results suggest that T-2 effects on mitochondrial respiration (PACE, 1983) and protein synthesis (PACE *et al.*, 1988) may be more significant as direct sites of action of the parent T-2 toxin than is cytoplasmic-ribosomal binding of the parent.

The nuclear fraction also contained measurable activity in the form of T-2 toxin. The persistence of T-2 and HT-2 in this subcellular fraction may contribute to the inhibition of DNA synthesis (UENO *et al.*, 1975; THOMPSON and WANNEMACHER, 1986).

These studies show that, while 93% of the radiolabel presented to the liver was metabolized and eliminated through bile and/or perfusate, at least 7% remained in the liver. Less than 1% was in the form of T-2 toxin. After initial uptake of T-2 toxin, radiolabel (T-2 and metabolites) was distributed to ribosomal, mitochondrial and nuclear fractions, the known sites of action of the trichothecene toxins. The predominant distribution of the

parent toxin was to mitochondria and nuclear fractions, suggesting a more direct affect of parent toxin on these organelles.

ACKNOWLEDGEMENTS

We thank Berthony Ladouceur and Charles Matson for their technical assistance, and Jackie Chaco-Farley for manuscript preparation.

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TABLE 1. PERCENT T-2 AND METABOLITES IN PERFUSATE

Time (min)		T-2	HT-2	3'OH HT-2	TRIOL	4DN*	TOL*	CONJUGATE
10	mean	21.0			29.3		19.0	26.0
	SEM	2.0			3.0		2.0	2.0
40	mean	6.0			26.0	19.2	14.2	27.1
	SEM	1.0			2.6	2.0	1.2	3.0
60	mean		23.0	21.1		6.0	16.4	31.0
	SEM		2.4	2.0		0.7	1.5	3.0

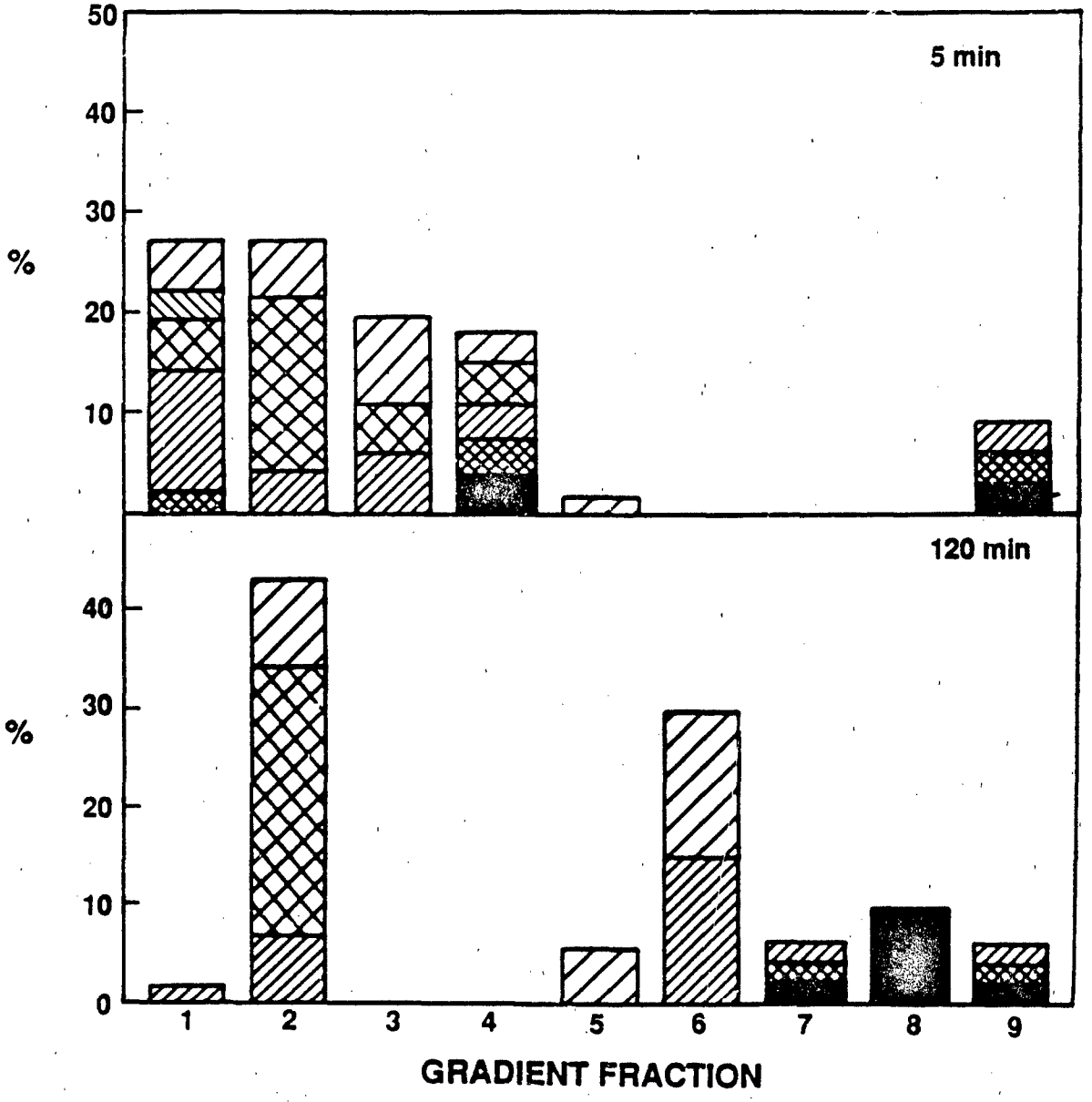
* 4DN, 4-deacetylneosolaniol; TOL, T-2 tetraol

TABLE 2. PERCENT DISTRIBUTION OF RADIOLABEL ASSOCIATED WITH SUBCELLULAR FRACTIONS

		Soluble	SER	Plasma Membranes		RER		Mitochondria		Nuclei
		1	2	3	4	5	6	7	8*	9
Time (min)										
5	mean	27.4	27.4	20.0	18.4	2.0				9.8
	SEM	4.9	4.8	10.8	9.4	1.8				7.7
15	mean		49.7	34.1	2.7					13.7
	SEM		9.5	6.1	2.5					0.9
30	mean	1.7	40.6			13.8	11.7	17.1	2.9	7.2
	SEM	1.5	9.1			12.6	6.2	7.6	1.8	2.9
60	mean		33.9			27.7	14.0	13.4	4.0	7.3
	SEM		10.7			11.1	2.0	1.7	0.1	4.2
120	mean	1.9	42.9			5.8	29.7	6.8	10.1	6.4
	SEM	1.8	6.4			5.5	2.1	6.8	2.8	1.4
Protein (mg)	mean	9.6	15.8	9.2	6.0	7.6	27.0	5.2	10.4	8.8
	SEM	1.1	1.0	2.0	1.3	1.3	4.0	0.7	1.7	1.3

*Mitochondria-rich fraction

FIG. 1. PERCENT DISTRIBUTION OF T-2 AND METABOLITES IN FRACTIONS OF LIVER HOMOGENATES AFTER A 5- OR 120-MIN PERFUSION OF [^{3}H]T-2. T-2, ■ ; HT-2, ▩ ; 4-deacetylneosolaniol, ▨ ; T-2 tetraol, ▩ ; unknown, ▧ ; and glucuronide conjugates, ▨ .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